Identification of small molecules that promote human embryonic stem cell self-renewal

（ヒト胚性幹（ES）細胞の未分化維持増殖を促進する低分子化合物の同定）

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Identification of small molecules that promote human embryonic stem cell self-renewal

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Abstract

Human embryonic stem cells (hESCs) and induced pluripotent cells have the potential to provide an unlimited source of tissues for regenerative medicine. For this purpose, development of defined/xeno-free culture systems under feeder-free conditions is essential for the expansion of hESCs. Most defined/xeno-free media for the culture of hESCs contain basic fibroblast growth factor (bFGF). Therefore, bFGF is thought to have an almost essential role for the expansion of hESCs in an undifferentiated state. Here, we report identification of small molecules, some of which were neurotransmitter antagonists (trimipramine and ethopropazine), which promote long-term hESC self-renewal without bFGF in the medium. The hESCs maintained high expression levels of pluripotency markers, had a normal karyotype after 20 passages, and could differentiate into all three germ layers.

Keywords: Human embryonic stem cell, screening, small molecule, basic fibroblast growth factor

1. Introduction

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have a self-renewal ability and pluripotency to differentiate into all three germ layers *in vitro* and *in vivo* [1-3]. Because of these notable properties, hESCs and hiPSCs are tools for basic biology, drug discovery research, and a cell source for regenerative medicine.

Undifferentiated hESCs and hiPSCs are usually maintained on mouse embryonic fibroblasts (MEFs) as feeders, or MEF-conditioned medium (CM) on Matrigel, which limits their clinical application owing to the potential risk of using animal components. In recent years, many commercial and non-commercial media have been reported to maintain hESCs and hiPSCs in culture under a feeder-free condition [4], but they have not been fully developed for the large-scale culture of cells because these media are expensive and often have batch-to-batch variations. One method to overcome such issues might be the addition of small molecules to the media as a replacement for growth factors and other components.

In this study, we aimed to identify small molecules to replace the role of basic

fibroblast growth factor (bFGF). Most defined/xeno-free media for hESCs contain bFGF at a higher concentration. Therefore, it is thought that bFGF is one of the most important components for robust expansion of hESCs in an undifferentiated state. Moreover, small molecules are not fully defined as replacements of bFGF for the expansion of undifferentiated hESCs in culture.

Here, we adopted a high-content screening (HCS) system using green fluorescent protein (GFP) expression regulated by the OCT4 promoter to monitor changes of cell fate in media. Furthermore, we focused on not only the intensity of GFP expression under the OCT4 promoter induced by each small molecule, but also the similarities in structure and the pharmacological effects of hit compounds. We found that selected small molecules support long-term hESC self-renewal in the absence of bFGF as evidenced by various pluripotency markers, a normal karyotype and differentiation into all three germ layers.

2. Materials and Methods

2.1 Construction of the hOCT4pro-EGFP reporter gene

We created an enhanced GFP (EGFP) reporter under the control of the human OCT4 promoter (hOCT4pro-EGFP) using a modified method from a previous report [5]. Briefly, the promoter region of human *OCT4* was cloned from the genomic DNA of KhES-1 cells by PCR using the following primers: forward, 5'-TTCCCATGTCAAGTAAGTGGGGTGG-3'; and reverse, 5'-ACCGGTGGGGAAGGAAGGCGCCCCAAGCC-3'. The PCR product was cloned into a pBSSK(-) vector and the sequence was confirmed by DNA sequencing. The human OCT4 promoter was digested with HindIII and AgeI, and then inserted into pEGFP-1 (Clontech).

2.2 Maintenance and transfection of hESCs

The hESC lines (KhES-1, KhES-3, and WA09 (H9)) were routinely cultured as described previously [6-8] on mitomycin C-treated MEF feeder cells in hESC medium consisting of DMEM/F12 (D-6421; Sigma) supplemented with 20% KnockOut Serum Replacement (KSR; Invitrogen), 0.1 mM non-essential amino acids (Sigma), 2 mM L-Glutamine (Sigma), 0.1 mM 2-mercaptoethanol (Sigma), and 5 ng/ml bFGF

(Wako, Japan). hESC medium without bFGF was used as hESC-basal medium (BM) in this study.

For feeder-free culture, hESCs were incubated with 2 mg/ml dispase (Invitrogen) in DMEM/F12 at 37°C for 10 min, and then detached using a cell scraper. Small clumps of hESCs were seeded onto Matrigel-coated plates in CM or hESC-BM with each compound. The plates were pre-coated with 0.2 mg/ml Matrigel (growth factor reduced; BD Biosciences) at 4°C overnight. The medium was removed, and the plates were washed with DMEM/F-12 to remove unbound Matrigel and then warmed to room temperature before use. CM was prepared as described previously [9] with the addition of 5 ng/ml bFGF.

Each compound, including small molecules from the Prestwick Chemical library, was added to hESC-BM at the indicated concentrations in the presence of 0.1% DMSO. Ethopropazine, promazine, retinoic acid (RA), PD98059 and trimipramine were purchased from Sigma, methotrimeprazine from Aurora Fine Chemicals LLC, and trimeprazine from the United States Pharmacopeial Convention.

Cell lines carrying hOCT4pro-EGFP were established by transfection with the

hOCT4pro-EGFP reporter plasmid. Before transfection, KhES-1 cells were seeded onto Matrigel-coated 100-mm tissue culture dishes in CM. ApaL1-linearized hOCT4pro-EGFP plasmid was transfected into KhES-1 cells using Fugene HD (Roche Diagnostics) according to the manufacturer's instructions. G418 selection (100 μg/ml) was applied at 24 hours after transfection. After about 14 days of selection, the surviving colonies were picked up individually and expanded as clones.

2.3 High-content screening

For HCS, cells cultured on feeder cells were treated with a CTK solution consisting of 1 mg/ml collagenase IV (Invitrogen), 0.25% trypsin (Invitrogen), 1 mM $CaCl_2$ and 20% KSR, and then detached as small clumps [6]. The cells were seeded in hESC-BM into 96-well plates (Greiner Bio-One), and from the following day, hESC-BM containing 2 μg/ml (~5 μM) of each small molecule from the Prestwick Chemical library (in the presence of 0.1% DMSO) was changed daily. Each compound was assessed in quadruplicate. Control wells containing 0.1% DMSO in hESC-BM were included on each plate. After 6 days of culture, cells were fixed with 4%

paraformaldehyde/PBS, washed with PBS, and then stained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). After three washes with PBS, fluorescence images were acquired by an ArrayScan-VTI System (Cellomics) and processed with the Target Activation BioApplication (Cellomics). To normalize data, a z-score based on the EGFP fluorescence intensity was calculated for each medium using the negative control medium (hESC-BM containing 0.1% DMSO).

2.4 Semi-quantitative PCR

Total RNA was extracted using an RNeasy Micro Kit (Qiagen), and then 0.5–1 μg total RNA was reverse transcribed with an Omniscript RT Kit (Qiagen) according to the manufacturer's instructions. For semi-quantitative PCR analysis, PCR was performed with TaKaRa ExTaq (TaKaRa, Japan). PCRs were optimized to allow semi-quantitative comparisons within the log phase of amplification. PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. Gene-specific primers are listed in Supplementary Table S1.

2.5 Teratoma formation assay

Approximately 2×10^6 cells were injected into the testes of severe combined immunodeficiency (SCID) mice (CLEA Japan). After 8 weeks, teratomas were surgically dissected from the mice, and then fixed in 4% paraformaldehyde/PBS. Samples were embedded in paraffin, sectioned at 5 μm and processed for hematoxylin and eosin staining. Animal protocols were approved by the Institutional Board on Animal Care at Kyoto University.

2.6 Statistical analysis

Data are shown as the average ± standard deviation (SD). Statistical significance was assessed using the Student's t-test. The probability level accepted for significance was $P < 0.05$.

2.7 Other methods

See Supplementary Methods for remaining methods including flow cytometric analysis, immunocytochemistry, Karyotype analysis, the EdU incorporation assay, the TUNEL assay and *in vitro* differentiation assay.

3. Results

3.1 High-throughput chemical screening to identify promotion of hESC self-renewal

To carry out a screen, we first established a human OCT4-GFP reporter system in hESCs, which contained –3917 to –1 base pairs relative to the transcriptional start site [5] (Fig. 1A). OCT4 is highly expressed in hESCs and downregulated upon differentiation. We initially isolated a 3D6 hESC clone, which showed GFP expression in an undifferentiated state, and was morphologically indistinguishable from the parental KhES1 cells (Fig. 1A). Furthermore, GFP expression in the cells was lost upon differentiation by 5 days of 10 μM RA treatment, as indicated by both fluorescence microscopy (Supplementary Fig. S1A) and flow cytometric analysis (Supplementary Fig. S1B). Moreover, flow cytometric analysis showed that OCT4-GFP expression was well correlated with OCT4 expression.

Undifferentiated 3D6 hESCs were seeded onto Matrigel-coated 96-well plates at a density of 3000 cells/well in hESC-BM. After overnight incubation, a compound

from the chemical library, as described in Section 2, was added to each well (2 μg/ml, ~5 μM). Medium containing compounds was changed daily for a further 5 days of incubation. Cells were analyzed for GFP expression using an Arrayscan VTI system (Cellomics).

Before performing our screening, to confirm whether the intensity of GFP fluorescence indicated the status of hESCs, we used CM, bFGF and mTeSR1 medium (StemCell Technologies) as controls for promotion of self-renewal, and RA or PD98059 as controls for induction of differentiation (Fig. 1B, and data not shown). The z'-factor is a parameter in statistics to assess the performance in high-throughput screening [10]. A z'-factor of > 0.5 was routinely obtained using this assay system, thereby supporting our conclusion that the intensity of GFP fluorescence using the hOCT4pro-EGFP reporter gene system could reliably identify small molecules that maintained hESCs in an undifferentiated state.

Using this assay, we screened 1120 compounds from Prestwick libraries, and 18 hit compounds (1.6% of the total chemicals screened; listed in Supplementary Table S2) that had effects on the self-renewal of hESCs were identified by exhibiting higher

OCT4-GFP expression by more than two SDs. Interestingly, we found that five out of the 18 chemicals were neurotransmitter antagonists that may share structural and physiological similarities, namely methotrimeprazine, trimipramine, trimeprazine, ethopropazine, and promethazine (Fig. 1C and D). In this study, we used trimipramine and ethopropazine for subsequent experiments. HCS using OCT4 immunostaining further showed that almost of the cells exhibited $OCT4^{high}$ in CM as a control for the undifferentiated state (98.9%). In contrast, DMSO strongly decreased the $OCT4^{high}$ population to 7.6%, whereas trimipramine and ethopropazine were effective for recovery of the $OCT4^{high}$ population to 41.0% and 16.1%, respectively (Fig. 2A). The effect became more noticeable over several passages. Both trimipramine and ethopropazine maintained KhES-3 and WA09 hESCs in an undifferentiated state, whereas DMSO treatment resulted in continuous differentiation of the cells (Fig. 2B). Although we initially screened these chemicals using KhES-1 hESCs, the cells were differentiated even in the presence of the chemicals after several passages (data not shown). In addition, OCT4-GFP or OCT4 expression in the cells, which was increased by the addition of chemicals to the medium, was still lower than that in cells cultured in

CM (Fig. 1D and 2A).

3.2 Effect of trimipramine and ethopropazine on long-term culture of hESCs

We found that two hESC lines (KhES-3 and WA09) cultured on Matrigel in hESC-BM containing trimipramine or ethopropazine proliferated for at least 20 passages without bFGF in the medium. Under these culture conditions, the cells showed compact colonies (Fig. 3A), which were similar to the undifferentiated colonies cultured in CM generally used as gold standard. We karyotyped 50 randomly selected cells by G-banding of both cell lines after 20 passages. Cells cultured with trimipramine or ethopropazine had normal karyotypes (Fig. S2A). Furthermore, RT-PCR analysis revealed that hESCs maintained high expression levels of pluripotency markers including OCT4 and NANOG, while the expression of differentiation marker genes was suppressed (Fig. 3B). Immunofluorescence analysis showed that the cells maintained the expression of pluripotency markers OCT4, NANOG and SOX2 (Fig. 3C). Flow cytometric analysis further indicated that the majority of hESCs cultured with these chemicals expressed pluripotency markers SSEA-3, SSEA-4, TRA-1-60, TRA-1-81,

and OCT4 with only low-level of expression of the differentiation marker SSEA-1 (Fig. 3D). Thus, trimipramine and ethopropazine may become substitutes for bFGF to maintain hESCs in an undifferentiated state. Furthermore, we found that trimipramine and ethopropazine treatments resulted in a similar proliferation rate of both hESC lines compared with those cultured in CM (Fig. 3E). We could not detect significant differences between trimipramine or ethopropazine in hESC-BM and CM in terms of cell cycling using a 5-ethyl-2'-deoxyuridine (EdU) uptake assay (S-phase) (Supplementary Fig. S2B). In addition, the chemicals did not induce apoptosis of hESCs using the TUNEL assay, although they showed insignificantly increased apoptosis of KhES-3 hESCs compared with those cultured in CM (Supplementary Fig. S2C). Together, we concluded that hESCs cultured with trimipramine or ethopropazine showed similar behavior compared with those cultured in CM.

Finally, we confirmed the pluripotency of hESCs that had been expanded in the presence of trimipramine or ethopropazine by examining their ability to form teratomas comprised of all three germ layers including neuroepithelium (ectoderm), intestinal epithelium (endoderm) and cartilage (mesoderm) (Fig. 4A). To assess the

pluripotency of hESCs *in vitro*, the cells were cultured in a differentiation medium described in the Supplementary Methods. We found that hESCs could differentiate into ectoderm (β-III tubulin), mesoderm (α-smooth muscle actin (SMA)) and endoderm (α-fetoprotein (AFP)) as determined by immunohistochemical analyses (Fig. 4B).

4. Discussion

Small molecules are different from proteins in terms of molecular size, and can penetrate multi-layer tissues easily. Therefore, small molecules are expected to be more efficient for maintenance of the undifferentiated state of hESCs and hiPSCs to subsequently induce specific differentiation. For example, Y-27632 has been identified as effective for maintaining the survival of dissociated hiPSCs [11]. Several small chemicals have also been identified to promote the generation of iPSCs from somatic cells [12-16]. Moreover, (-)-indolactam V has been found to promote pancreatic differentiation of human pluripotent cells [17]. Here, we show that some neurotransmitter antagonists support the expansion of undifferentiated hESCs without bFGF in the medium.

Although a previous study using a HCS system identified small molecules that regulate undifferentiated proliferation of hESCs, the small molecules used in the study cannot support long-term culture [18]. In this study, we first addressed following points to develop a more reliable HCS system for hESCs. Previous HCS systems evaluate the undifferentiated state of hESCs by the expression of OCT4 or SSEA-3 using immunohistochemistry [18-20]. Instead, we used a reporter gene system consisting of GFP regulated by the hOCT4 promoter, which enables evaluation of OCT4 expression in cells directly without immunostaining procedures. Furthermore, we assessed not only the score of the effect of each small molecule, but also their similarities in terms of structure and pharmacological effects for hit compounds previously proposed by Lukaszewicz et al. [21]. This strategy may enable more reliable HCS for the identification of small molecules that drive hESC self-renewal as a replacement for bFGF.

In this study, we show that trimipramine and ethopropazine permit long-term hESC self-renewal without bFGF in the medium. We examined three hESCs lines (KhES-1, KhES-3, and WA09) and found that KhES-3 and WA09, but not KhES-1,

hESCs successfully maintain undifferentiated expansion in the presence of these small molecules. Indeed, as indicated in Figs. 1D and 2A, OCT4 expression increased in the presence of the small molecules compared with that of DMSO, but OCT4 expression was still lower than that in CM. Therefore, the small molecules were not fully effective for the maintenance of undifferentiated proliferation of some hESC lines. We examined higher concentrations of the small molecules, but they showed cytotoxicity at such concentrations (data not shown). Taken together, further improvement of the small molecules to reduce their cytotoxicity while increasing the self-renewal of many hESCs lines needs to be achieved for complete replacement of bFGF in media.

The FGF pathway is activated by FGF ligands binding to FGF receptors, which in turn may trigger activation of various downstream signaling pathways including MAP kinase, PI3K kinase/AKT and PLC/PKC pathways [22]. However, the molecular mechanism by which bFGF promotes undifferentiated expansion of hESCs is still unclear. A MAP kinase kinase (MEK) inhibitor, PD0325901, is reported to be effective for hESC maintenance [23]. Activation of the PLC/PKC pathway may induce hESC differentiation [24]. PIK3/AKT signaling activity has been reported to participate

in undifferentiated proliferation by suppression of MAP and canonical Wnt signaling pathways [25]. Therefore, the PIK3/AKT pathway may be a potential target of bFGF signaling. However, it remains unclear whether such small molecules related to PIK3/AKT pathways are ideal replacements for bFGF. Thus, small molecules related to growth factors and their signaling pathways have not been fully identified for the replacement of bFGF to expand undifferentiated hESCs in culture.

In this study, we showed that trimipramine and ethopropazine permit long-term hESC self-renewal without bFGF in the medium. Further studies will clarify the molecular mechanisms of trimipramine and ethopropazine, which enable long-term expansion of hESCs, including the interactions between these chemicals and the FGF or PIK3/AKT pathways.

For feeder-free culture systems, CM is usually used as the current gold standard. In this study, we showed that trimipramine and ethopropazine treatments resulted in similar growth curves and cell cycles as those of hESCs cultured in CM. Thus, the small molecules enabled similar performance compared with that of CM for the expansion of undifferentiated hESCs.

In conclusion, we established a HCS platform to identify small chemicals that promote hESC self-renewal. Using the screening system, we identified trimipramine and ethopropazine as novel small molecules that promote long-term hESC self-renewal without bFGF in the medium. This screening system may help to identify more small molecules to achieve robust proliferation of hESCs and hiPSCs in low-cost and growth factor-free medium.

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Figure Legends

Fig. 1. High-content screening system and characterization of hit compounds.

(A) (Left) Vector map of hOCT4pro-EGFP. (Right) Phase contrast and fluorescence images of parental KhES-1 and hOCT4pro-EGFP transfectant (3D6 clone) hESCs cultured on MEF feeders. Bar indicates 200 μm. (B) (upper) Representative fluorescence image of EGFP in 3D6 clones cultured in various media (CM, hESC-BM containing 0.1% DMSO, and hESC-BM containing 10 μM RA) for 5 days. Bar indicates 500 μm. (lower) Calculation of GFP intensity in upper fluorescence images. A.U. corresponds to arbitrary unit. Data are presented as the means ± SD. (C) Five of the 18 hit small molecules from the 1120-chemical library. The small molecules shared a z-score of higher than 2 SD, as well as structural and physiological similarities. (D) Representative fluorescence images of 3D6 hESCs cultured in hESC-BM with the five hit compounds. Expression of markers of the undifferentiated state was observed by EGFP reporter gene expression driven by the hOCT4 promoter (hOCTpro-EGFP: green) in immunocytochemistry. Cells were identified by DAPI (blue). Bar indicates 500 μm. CM, conditioned medium.

Fig. 2. Small molecules support short-term culture of hESCs without bFGF in the medium.

(A) Histograms of the intensity of OCT4 immunostaining in hESCs treated with CM, DMSO, or small chemicals. (B) Morphology of KhES-3 and WA09 hESCs cultured in hESC-BM with trimipramine or ethopropazine after several passages, respectively. DMSO caused differentiation of hESCs, whereas CM and small molecule-containing media maintained the undifferentiated state of hESCs.

Fig. 3. Small molecules support long-term culture of undifferentiated hESCs without bFGF in the medium.

(A) Phase contrast micrographs of KhES-3 and WA09 hESCs cultured in hESC-BM with trimipramine or ethopropazine after 25 passages. Bar indicates 200 μm. (B) Expression of pluripotency marker genes (*OCT4* and *NANOG*) and differentiation marker genes (ectoderm: *LHX2* and *DLK1*; endoderm: *GATA6* and *GATA4*; mesoderm; *CDH5* and *FOXF1*; trophectoderm: *CDX2* and *CGA*) was analyzed by RT-PCR. (C)

Immunocytochemical detection of pluripotency marker genes (OCT4, SOX2, and NANOG) in KhES-3 and WA09 hESCs cultured in hESC-BM with trimipramine or ethopropazine after 40 passages. Bar indicates 100 μm. (D) Flow cytometric analysis of markers of the undifferentiated state (OCT4, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81) and a differentiation marker (SSEA-1) after 40 passages of culture in CM or hESC-BM with trimipramine or ethopropazine. (E) Growth rates of KhES-3 and WA09 hESCs cultured in CM or hESC-BM with trimipramine or ethopropazine during 4 days of culture after seeding. Cell numbers were counted every 24 hours. The data represent means ± SD.

Fig. 4. Small molecules sustain the pluripotency of KhES-3 and WA09 hESCs without bFGF in the medium.

(A) Characterization of teratomas derived from KhES-3 and WA09 hESCs cultured with trimipramine or ethopropazine. Hematoxylin and eosin staining of paraffin-embedded teratoma sections identified hESC differentiation into various tissues including neural pigment (ectoderm), a gut-like tube (endoderm), and cartilage

(mesoderm). Bar indicates 100 μm. (B) Immunostaining for markers of the three germ layers in differentiating KhES-3 and WA09 hESCs cultured with hit compounds after 40 passages: ectoderm (βIII-tubulin), endoderm (AFP), and mesoderm (α-SMA).

Supplementary Fig. S1

Comparative expression of the hOCT4pro-EGFP reporter between undifferentiated and differentiated states.

(A) KhES-1 cells carrying hOCT4pro-EGFP (3D6 clone) were cultured in CM (undifferentiated state) or 10 μM RA (differentiated state) for 5 days on Matrigel. **(B)** Co-expression of OCT4 and EGFP was analyzed in undifferentiated and differentiated states by flow cytometric analysis.

Supplementary Fig. S2

Small molecules sustain the pluripotency of hESCs.

(A) KhES-3 and WA09 hESCs cultured with trimipramine or ethopropazine (after 20 passages) had a normal karyotype as shown by G-banding analysis. **(B)** Cell cycle analysis of KhES-3 and WA09 hESCs cultured in CM or hESC-BM with hit compounds. The number of cells that incorporated EdU was detected and enumerated by labeling with Alexa Fluor 488 after 4 days of culture. The data represent the means ± SD of three independent experiments. **(C)** After 4 days of culture, apoptotic KhES-3

and WA09 cells in CM or hESC-BM with hit compounds were analyzed by a TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay. The data represent the means ± SD of three independent experiments.

Supplementary Table S1

List of primers used for RT-PCR in this study.

Supplementary Table S2

List of 18 hit small molecules with z-scores of more than 2 SD.

Supplementary Methods

Flow cytometric analysis

Cells were dissociated with PBS containing 0.2% EDTA for 2–5 minutes at room temperature, followed by treatment with 0.05% trypsin/EDTA for 1 minute at 37°C. For analysis of cell surface markers, 2×10^5 cells in a single cell suspension were incubated with a primary antibody diluted in working solution (0.1% BSA/PBS) for 30 minutes at 4°C, washed three times with working solution, and then incubated with a secondary antibody diluted in working solution for 30 minutes at 4°C. Cells were counterstained with 1 μg/ml propidium iodide (Invitrogen) just prior to analysis. For analysis of OCT4, cells were fixed with 2% paraformaldehyde/PBS for 30 minutes at 4°C, and then permeabilized with SPB buffer (1 mg/ml saponin and 1% BSA/PBS). Cells were incubated with the primary antibody against OCT4, and then a secondary antibody. Flow cytometric analysis was performed using a FACSCalibur and the CellQuest software package (Becton Dickinson). Primary antibodies against the following markers were used: SSEA-1 (2 μg/ml; Developmental Studies Hybridoma Bank (DSHB)), SSEA-3 (2 μg/ml; DSHB), SSEA-4 (1 μg/ml; DSHB), TRA-1-60 (1 μg/ml; Abcam),

TRA-1-81 (1 μg/ml; Abcam), TRA-2-54 (1 μg/ml; DSHB) and OCT4 (1 μg/ml; Santa Cruz Biotechnology). Secondary antibodies used were as follows: FITC-conjugated anti-mouse IgG (10 μg/ml; Becton Dickinson), Alexa Fluor 488-conjugated anti-mouse IgG (2 μg/ml; Molecular Probes), Alexa Fluor 488-conjugated anti-rat IgM (4 μg/ml; Molecular Probes), or Alexa Fluor 647-conjugated anti-mouse IgG (2 μg/ml; Molecular Probes).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde/PBS for 20 minutes at room temperature. After three washes with PBS, the cells were permeabilized with 0.2% Triton X-100/PBS for 5 minutes, and blocked with 5% BSA/PBS for 1 hour at room temperature. The cells were then incubated with the primary antibody (diluted in 1% BSA/PBS) at 4°C overnight, and subsequently with the secondary antibody (diluted in 1% BSA/PBS) for 1 hour at room temperature. Nuclei were counterstained with 1 μg/ml DAPI for 5 minutes at room temperature. Fluorescence staining was observed by a Zeiss imaging system with AxioVision software (Zeiss). Primary antibodies were OCT4

(200 ng/ml; Santa Cruz Biotechnology), NANOG (400 ng/ml; ReproCell, Japan or 1:100; Cell Signaling Technology), SOX2 (2.5 μg/ml; R&D Systems). The secondary antibodies used were Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 546-conjugated goat anti-rabbit IgG, or Alexa Fluor 647-conjugated goat anti-rabbit IgG (4 μg/ml; Molecular Probes).

Karyotype analysis

Chromosome spreads were prepared as described previously [7]. Briefly, cells were treated with KaryoMAX-COLCEMID Solution (100 ng/ml colcemid; Invitrogen) for 2 hours, trypsinized, incubated in 0.075 M KCl, and then fixed in Carnoy's fixative. The cells were spread onto glass slides and stained with Giemsa. Chromosome spreads were analyzed by randomly selecting 50 cells using the Ikaros Karyotyping System (META system).

EdU incorporation assay

To measure cell proliferation, hESCs grown on Matrigel-coated plates were labeled

with 10 μM EdU (Invitrogen) for 1 hour. Cell fixation, permeabilization, and EdU detection were performed following the manufacturer's instructions for the Click-iT EdU Alexa Fluor 488 HCS Assay (Invitrogen). Fluorescence images were acquired by an ArrayScan VTI System and processed by the Target Activation BioApplication (Cellomics).

TUNEL assay

For TUNEL staining, hESCs grown on Matrigel-coated plates were fixed with 4% paraformaldehyde/PBS, and then subjected to a TUNEL assay using a DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. Fluorescence images were acquired by an ArrayScan VTI System and processed by the Target Activation BioApplication

Embryoid body-mediated differentiation assay

For embryoid body formation, cells were harvested by treatment with a dispase solution followed by cell scraping. For immunocytochemistry, small clumps of hESCs were

transferred to a petri dish in differentiation medium and cultured for 4 days, and then transferred onto Matrigel-coated glass slides (BD Biosciences), and followed by an additional 7 days of culture. Differentiation medium consisted of DMEM supplemented with 20% FBS, 0.1 mM non-essential amino acids, 1 mM L-glutamine and 0.1 mM 2-mercaptoethanol. Primary antibodies against βIII-tubulin (1:200; Sigma), AFP (1:200; Sigma), and αSMA (1:200; Sigma) were used. The secondary antibody used was an Alexa Fluor 555-conjugated goat anti-mouse IgG (4 μg/ml; Molecular Probes).

Figure 1

Fig. 1

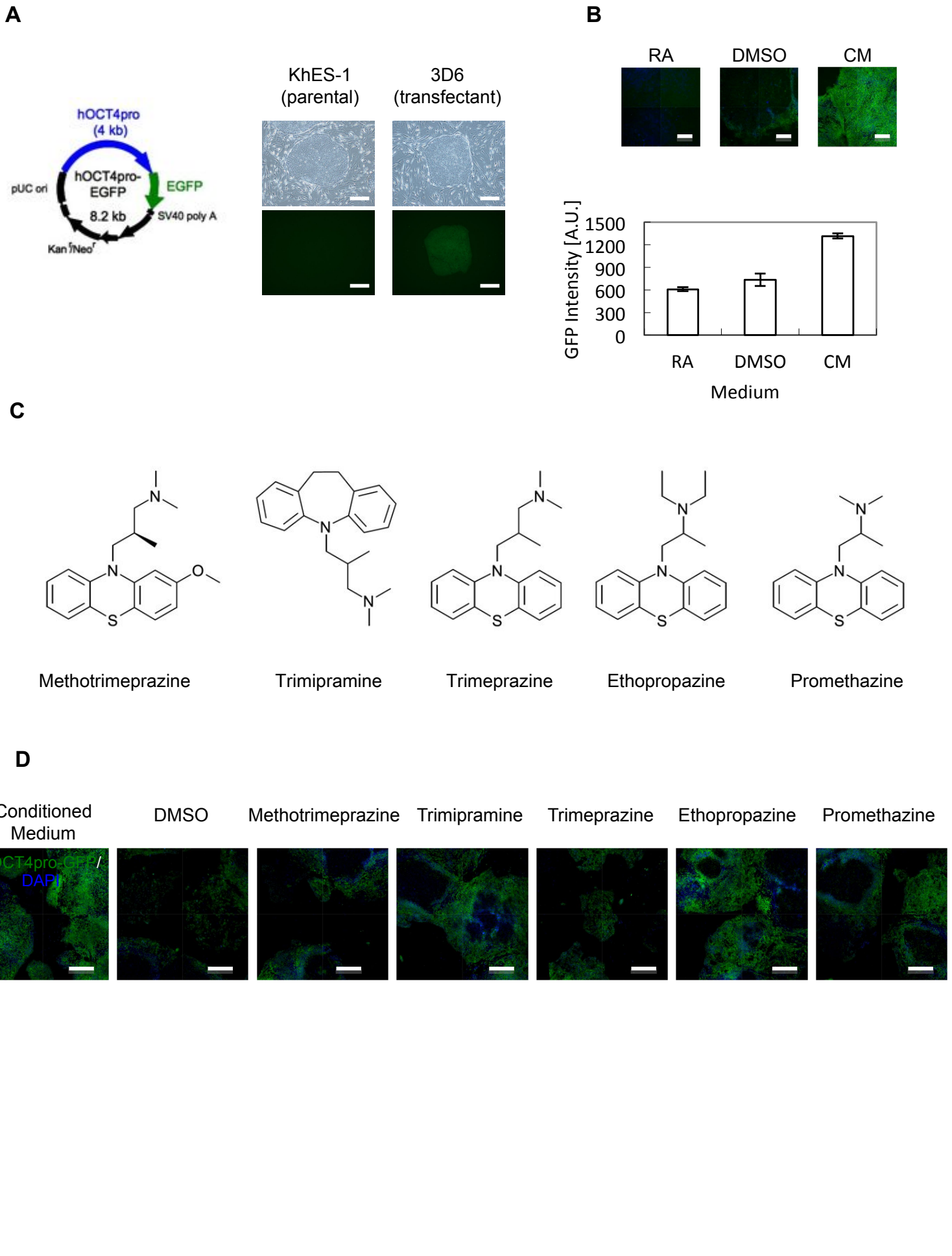
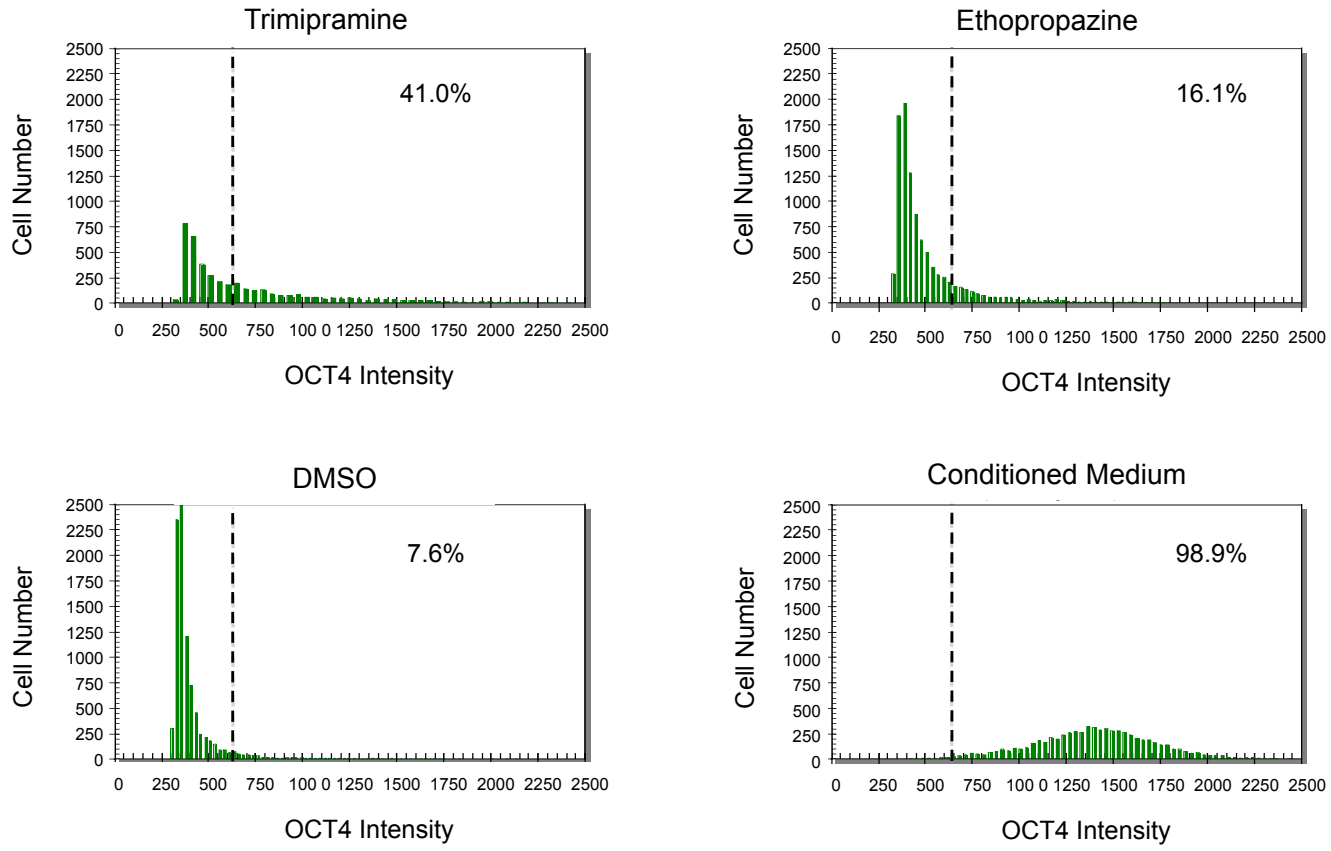


Figure 2

Fig. 2

A



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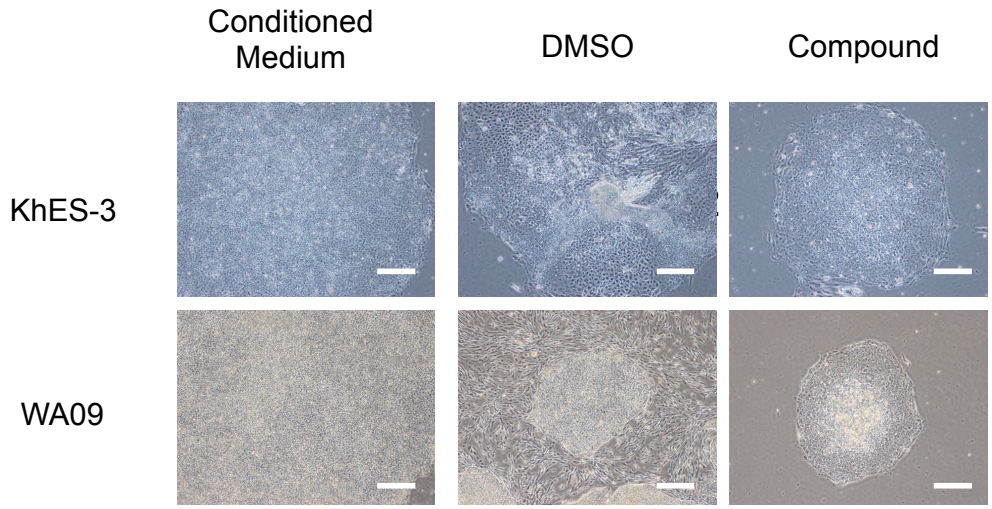


Figure 3

Fig. 3

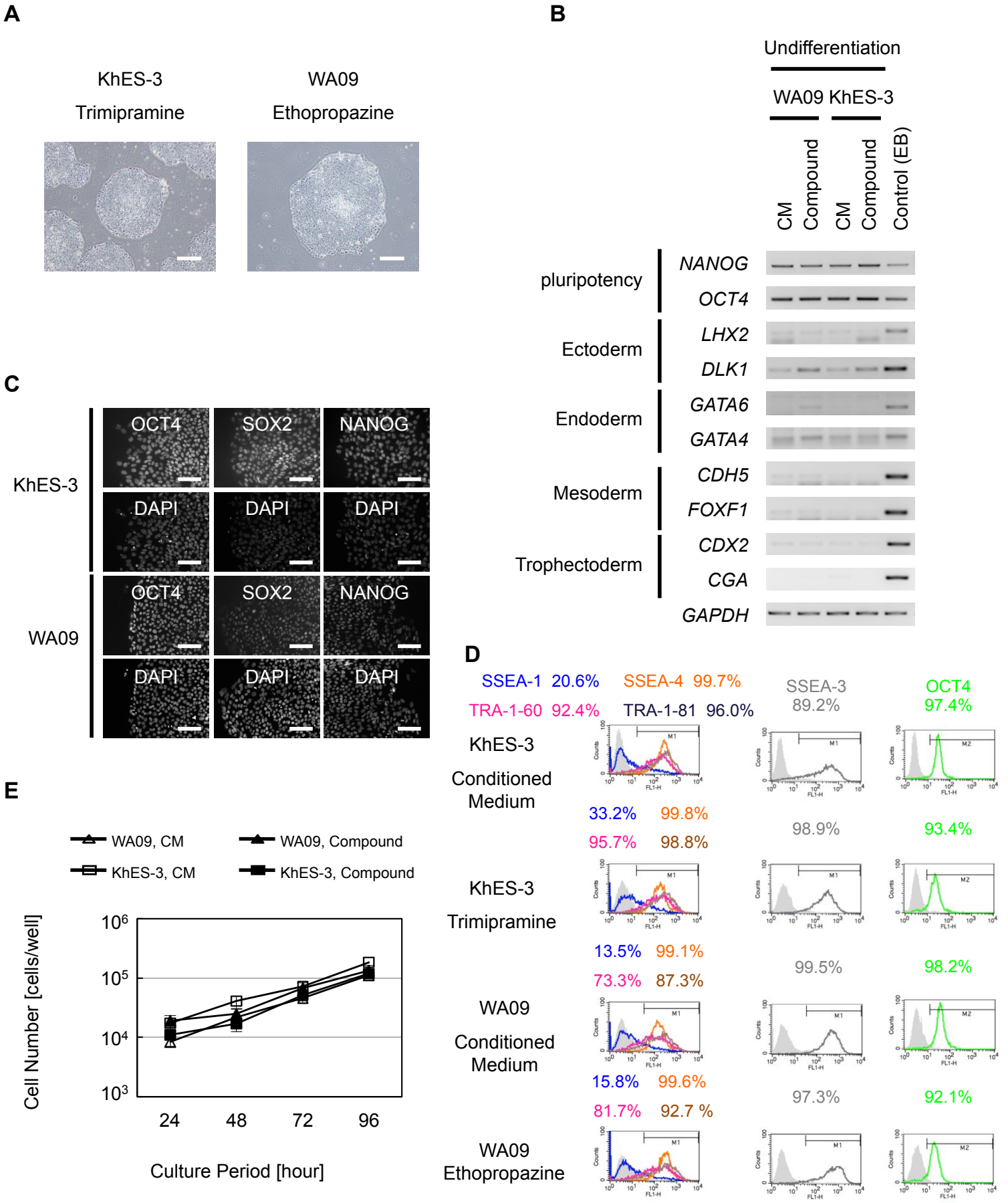
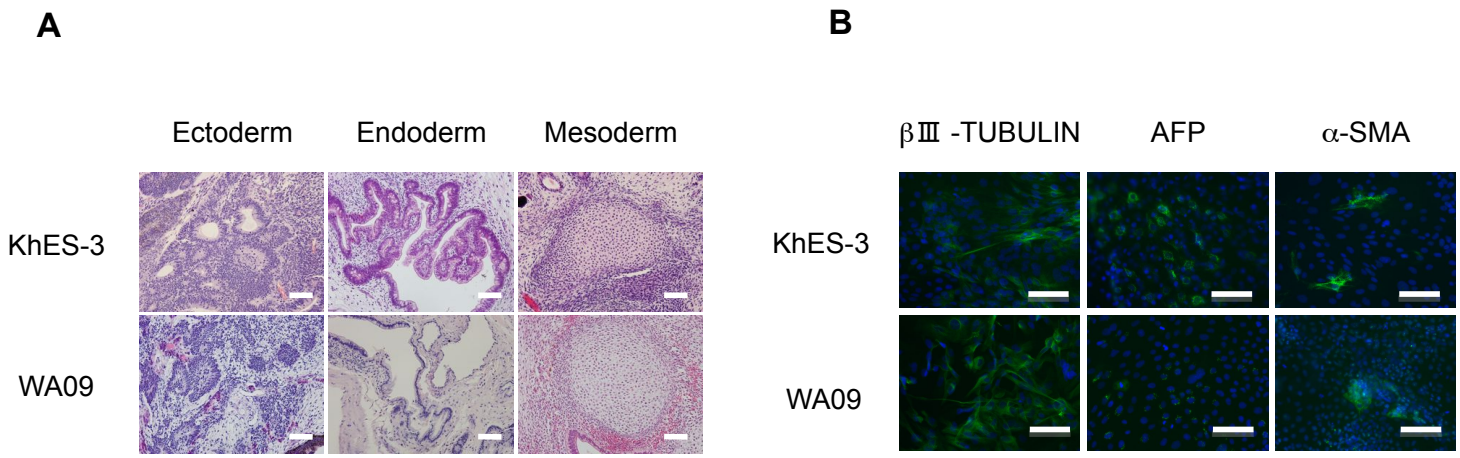


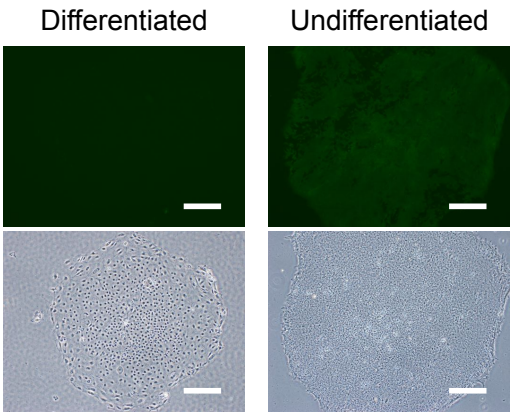
Figure 4

Fig. 4



Supplementary Fig. S1

A



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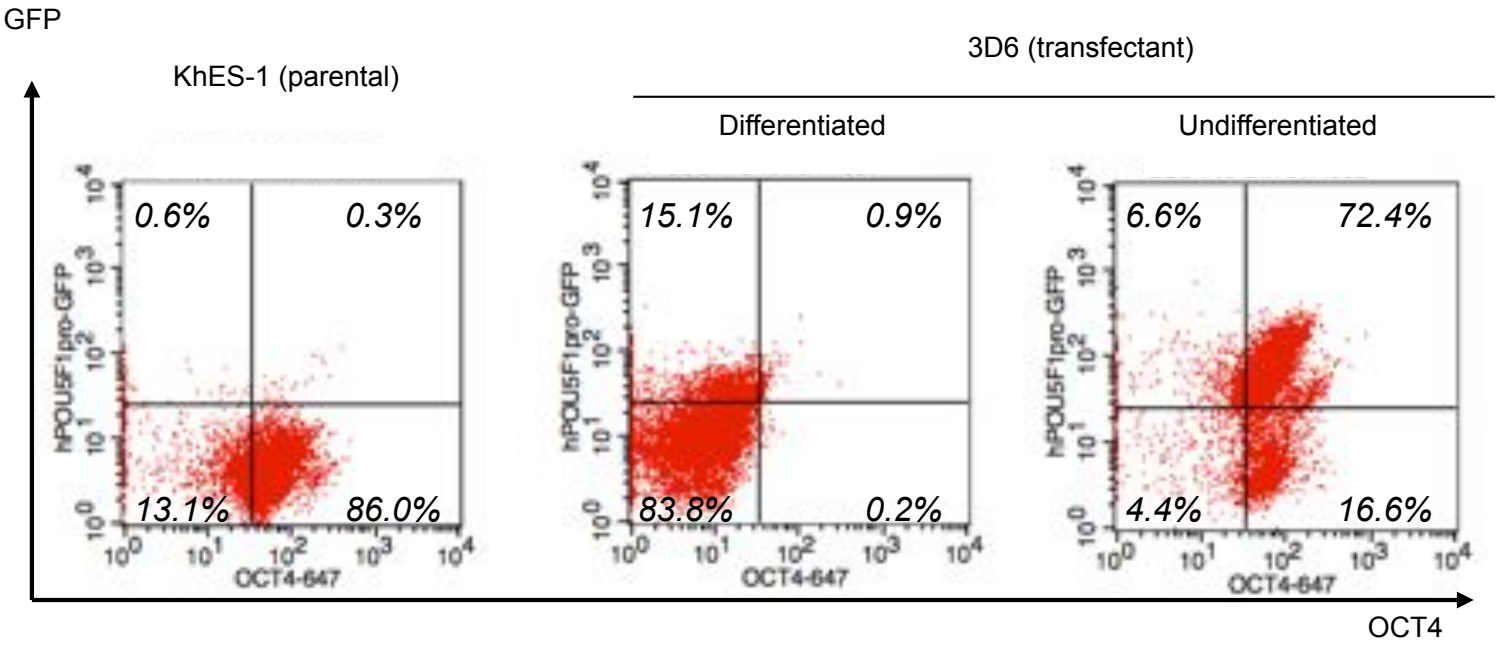
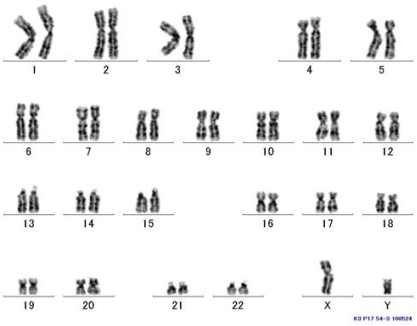


Supplementary Fig. S2

A

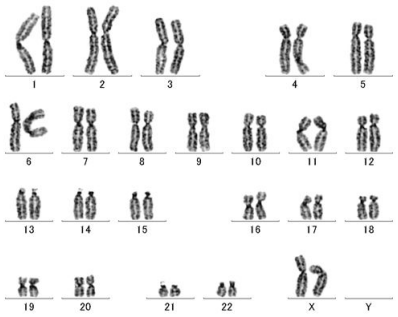
KhES-3

Trimipramine



WA09

Ethopropazine



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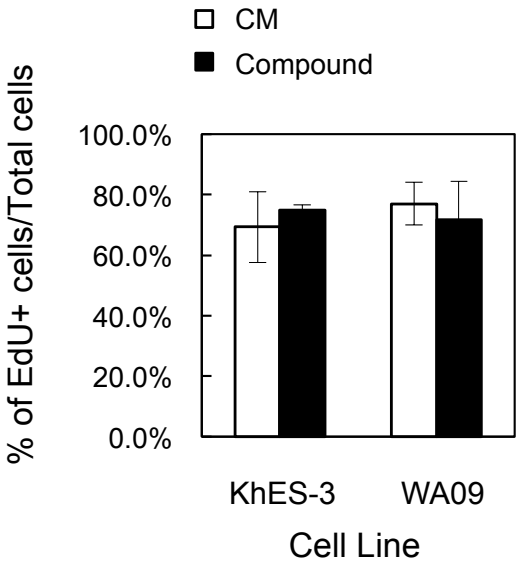

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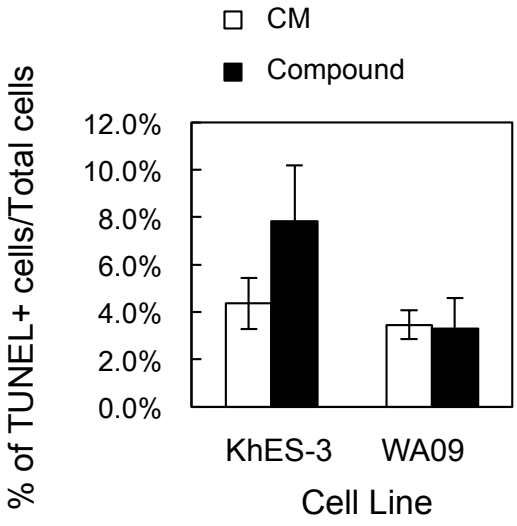

Supplementary Table S1

Genes	Forward primer	Reverse primer	Product	Annealing
	5'-3'	5'-3'	(bp)	(°C)
NANOG	AAGACAAGGTCCCGGTCAAG	CCTAGTGGTCTGCTGTATTAC	584	58
OCT4	GAGAACAATGAGAACCTTCAGGAGA	TTCTGGCGCCGGTTACAGAACCA	218	58
LHX2	CCAAGGACTTGAAGCAGCTC	TGTTTTCCTGCCGTAAGAGG	114	60
DLK1	GACGGGGAGCTCTGTGATAG	GGGCACAGGAGCATTCATAG	109	60
GATA6	TGTGCAATGCTTGTGGACTC	AGTTGGAGTCATGGGAATGG	161	60
GATA4	CCTGGCCTGTCATCTCACTAC	AGAGGACAGGGTGGATGGA	100	60
CDH5	GATCAAGTCAAGCGTGAGTCG	AGCCTCTCAATGGCGAACAC	114	60
FOXF1	ACAGCGGCGCCTCTTATATC	CTCCTTTCGGTCACACATGC	194	60
CDX2	CCGAACAGGGACTTGTTTAGAG	CTCTGGCTTGGATGTTACACAG	198	60
CGA	GTTTCTGCATGTTCTCCATTC	GTGGACTCTGAGGTGACGT	195	60
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226	60

Supplementary Table S2

Chemical name	fmla structure	mol weight structure	CAS number
Quinacrine dihydrochloride dihydrate	C23H36Cl3N3O3	508.9	6151-30-0
Harmalol hydrochloride dihydrate	C12H17ClN2O3	272.7	6028-07-5
Boldine	C19H21NO4	327.4	476-70-0
Meclofenoxate hydrochloride	C12H17Cl2NO3	294.2	3685-84-5
Fenoprofen calcium salt dihydrate	C45H43CaO11	799.9	53746-45-5
Methotrimeprazine maleate salt (Methotrimeprazine)	C23H28N2O5S	444.6	7104-38-3
Trimipramine maleate salt (Trimipramine)	C24H30N2O4	410.5	521-78-8
Trimeprazine tartrate (Trimeprazine)	C58H72N6O6S3	1045.4	4330-99-8
Sulfaphenazole	C15H14N4O2S	314.4	526-08-9
Doxylamine succinate	C21H28N2O5	388.5	562-10-7
Dehydrocholic acid	C24H34O5	402.5	81-23-2
Pargyline hydrochloride	C11H14ClN	195.7	306-07-0
Cloxacillin sodium salt	C19H17ClN3NaO5S	457.9	642-78-4
Ethopropazine hydrochloride (Ethopropazine)	C19H25ClN2S	348.9	1094-08-2
Promethazine hydrochloride (Promethazine)	C17H21ClN2S	320.9	58-33-3
Crotamiton	C13H17NO	203.3	483-63-6
(S)-propranolol hydrochloride	C16H22ClNO2	295.8	4199-10-4
Quinic acid	C7H12O6	192.2	36413-60-2